REMARKS

Claims 28-36 were pending in the present application. By this Amendment, Applicants have canceled claims 29 and 31 without prejudice and have incorporated subject matter found therein into claim 28. Applicants have amended claims 30 and 32 to make their dependencies consistent with the above claim amendments and cancellations. The present Amendment introduces no new matter and thus, its entry is respectfully requested. Upon entry of the present Amendment, claims 28, 30, and 32-36 will be pending and under examination.

The July 27, 2004 Office Action

Examiner's Claim Rejections - 35 USC § 112, first paragraph, enablement

The Examiner rejected claims 28-30 and 32-36 under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement. According to the Examiner, the identification of new proteasome inhibitors based upon a crystallized fraction of a cell extract would require undue experimentation. The Examiner acknowledged the specification's teachings that the identification and isolation of new proteasome inhibitors is carried out using various specifically recited known computer-aided modeling programs. The Examiner then stated that because the claims themselves do not specifically recite these modeling steps, the claims are not enabled for identifying inhibitors. More particularly, the Examiner stated that "[o]ne of skill in the art would not know how to go from the step of analyzing to identification of inhibitors without additional

steps that include computer modeling such as outlined in the specification without undue experimentation."

In response, Applicants respectfully disagree with the Examiner's position. First, Applicants reiterate that the novel purification process of the present invention results in proteasomes sufficiently pure to obtain crystals, and from these crystals, routine procedures known in the art and described in the specification can readily be used by one of ordinary skill to identify inhibitors to the proteasomes. The acknowledgment by the Examiner that the specification indeed provides a description of how one can analyze the crystal structures to identify inhibitors using computer-aided modeling is itself evidence of enablement. Applicants believe that the Examiner is incorrect in requiring the modeling steps to be specifically recited in the claims. It is clear, particularly from pages 7-8 of the specification, that computer-aided modeling is a preferred method of "analyzing the structure of the resulting crystals." It is improper to limit an Applicant to preferred embodiments in the absence of limiting prior art, and thus, there does not appear to be a need to include these specific modeling steps in the claim itself to satisfy the enablement requirement. Nevertheless, without conceding the correctness of the Examiner's position, but to expedite allowance of the subject application, Applicants have canceled claims 29 and 31, and have amended claim 28 to recite computer-aided modeling. Accordingly, this aspect of the Examiner's enablement rejection has been fully overcome.

The Examiner also asserted that the working examples pertaining to protein preparations from yeast do not provide enablement for the full scope of the claims, which refer to the use of "eukaryotic cells" as the method's starting material.

Applicants respectfully traverse this aspect of the Examiner's rejection. The structures of yeast proteasomes are sufficiently similar to those of humans and other eukaryotes so that the process can be, and in fact has been, employed successfully with at least both yeast and human proteasomes. In that regard, Applicants attach hereto a copy of a recent publication, Furet, et al. J. Med. Chem. 47:4810-4813 (2004), which describes how others followed the teachings of the present invention to identify proteasome inhibitors, and thus strongly supports Applicants' position that the present claims are fully enabled by the teachings set forth in the specification. In particular, inhibitors of human 20S proteasome were obtained using the X-ray crystal structure of the yeast proteasome provided by the present invention. Applicants direct the Examiner's attention to page 4810 of the Furet publication (at column 2, second paragraph from the bottom) which specifically refers to the method of the claimed invention, as described in a paper by inventor Groll, et al. (see FN 26). (The Groll paper is already of record in the present case, as having been cited in a previously filed IDS). The attached Furet paper confirms that by using the X-ray crystal structure of the yeast proteasome, obtained according to the purification process of the claimed invention, a homology model of the active site for the chymotrypsin-like proteolytic activity could be obtained, namely for the human 20S proteasome. Furthermore, Furet, et al. report that chemical compounds can be identified as inhibitors of the human 20S proteasome

simply by following the teachings of the present application. (Furet, page 4811, column 2). In short, the data presented in the Furet publication, along with the inhibitors identified therein clearly demonstrate that the claimed method of the present invention can be and in fact has been successfully used for both human and yeast proteasomes. The present claims therefore clearly are fully enabled by the specification and thus, Applicants respectfully request that the Examiner reconsider and withdraw this aspect of the rejection.

The Examiner also indicated that the "analyzing" step is not sufficient to provide guidance as to what model should be used to identify the proteasome inhibitors.

In response, Applicants respectfully disagree. Applicants assert that the amendments and arguments presented above address this aspect of the rejection as well. Moreover, Applicants point out that the specification does in fact provide ample guidance to one of ordinary skill in the art, as evidenced by the disclosure at pages 7-8, as to what structural features one should look to when analyzing the proteasome crystals for the design of inhibitors. Applicants therefore request withdrawal of this aspect of the rejection.

The Examiner also questioned how inhibitors can be identified without including the step, included in Example 2 (page 13), of immersing the crystal in an inhibitor solution.

In response, Applicants respectfully traverse this aspect of the Examiner's rejection. As is clear from the specification (see, for example, page 13), such a step is not required to identify inhibitors, but merely represents an alternate embodiment of the process. As the specification shows, one crystallization was carried out without an inhibitor, and two additional

crystallizations, each with different inhibitors included, were also carried out. Accordingly, Applicants respectfully request withdrawal of this aspect of the rejection.

Applicants believe, particularly in light of the present amendments and the evidence presented in the Furet, et al. publication, that all of the Examiner's concerns have been fully addressed and that the present claims are fully enabled. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the enablement rejection of claims 28-30 and 32-36 under 35 U.S.C. §112, first paragraph.

Examiner's rejection under 35 U.S.C. §112, first paragraph, written description

The Examiner rejected claim 36 under 35 U.S.C. §112, first paragraph, as allegedly lacking adequate written description. The Examiner asserted that the claim, which is directed to a "proteasome inhibitor identified by the method of claim 28" does not find adequate support in the specification because no inhibitor identified by the method is disclosed.

In response, Applicants respectfully traverse this rejection. The specification clearly describes both how to obtain inhibitors using the present method and what characteristics define such inhibitors (see, e.g. pages 7-8) obtained by the method. As noted above, several such inhibitors were also obtained by Furet following the teachings of the present invention. Applicants therefore respectfully request that the written description rejection be reconsidered and withdrawn by the Examiner.

Examiner's rejection under 35 U.S.C. §112, second paragraph

The Examiner rejected claims 28-36 under 35 U.S.C. §112, second paragraph, as allegedly being indefinite. Specifically, the Examiner asserted that the step of "analyzing the structure of the resulting crystals" in claim 28 is unclear, given that the term "analyzing" can mean any number of techniques, including, for example, x-ray diffraction, electron microscopy, and determination of 3D coordinates. The use of the phrase "analyzing the structure of the resulting crystals to identify new proteasome inhibitors" is clear, particularly in light of the description in the specification at pages 7-8, which provides ample guidance to one of skill in the art on how to design inhibitors based on the structure of the crystals obtained by the purification process. In light of this description and further in light of the amendments and remarks made herein, one of ordinary skill in the art would have no difficulty understanding the meaning of the term "analyzing " as it is now used in the claims.

The Examiner also rejected claim 30 as vague and indefinite, asserting that it is unclear from where the crystal data comes. Specifically, the Examiner inquired whether the proteasome pockets S1 of the recited subunits are from yeast or another eukaryote, and added that it is unclear how a proteasome pocket from one species could be the same in another species.

In response, Applicants point out that, consistent with the report set forth in the Furet paper, the structures of yeast, human, and other eukaryotic proteasomes, including the presence of the β-subunits, are similar. Accordingly, one of skill in the art would fully understand the use of all terms in the claims referred to by the Examiner.

The Examiner also rejected claim 32 as vague and indefinite, asserting that it is unclear how modification of the crystal data of a yeast proteasome is performed. Applicants first refer to the discussion concerning the enablement rejection above, including the presentation of the Furet paper. Furthermore, the specification (at page 8) indicates that the modification involves a homology modeling carried out by a known molecular graphic program (such as O, INSIGHT, or FRODO) for the purpose of designing human inhibitors based on the structural data from the yeast proteasomes. Claim 32, therefore, is clear as written, given this description in the specification.

Accordingly, all claims meet the requirements of 35 U.S.C. §112, second paragraph, and Applicants therefore respectfully request that the Examiner reconsider and withdraw the above indefiniteness rejections.

In view of the above remarks and amendments, Applicants believe that all of the Examiner's rejections as set forth in the July 27, 2004 Office Action have been fully overcome and that the present application is in condition for allowance. The Examiner is invited to telephone the undersigned if it is deemed to expedite allowance of the application.

No fee is believed due in connection with the filing of this Amendment. However, if any fee is deemed necessary, authorization is hereby given to charge such fee, or credit any overpayment to Deposit Account No. 02-2135.

Respectfully submitted,

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Attachment: Furet publication

2923-130.am2.wpd

Entry into a New Class of Potent Proteasome Inhibitors Having High Antiproliferative Activity by Structure-Based Design

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Abstract: Proteasome inhibition is a therapeutic concept of current interest in anticancer research. We report here the design, synthesis, and biological characterization of prototypes of a new class of noncovalent proteasome inhibitors showing high activity in biochemical and cellular assays.

The proteasome is a multicatalytic protease complex that degrades intracellular proteins tagged for destruction by the covalent attachment of multiple ubiquitin molecules.[1,2] The ubiquitin−proteasome system is involved in the degradation of key components of the molecular machinery on which rely such important cellular functions as transcription, cell-cycle progression, tumor suppression, and apoptosis.[3−8] Given the wide range of substrates and processes that are regulated by this system, the components of the ubiquitin−proteasome pathway have become the focus of intensive biochemical research, especially in the oncology area. Although this pathway can be blocked at various steps, most inhibitors have been designed to target the proteolytic activities of the proteasome. Thus, it was shown that inhibition of proteasomal activity induced cell-cycle arrest and apoptosis in tumor cells, thereby leading to antiproliferative effects.[9−11] The observation that malignant cells were more susceptible to the proapoptotic effects of proteasome inhibition than normal cells raised the notion of proteasome inhibition as a potential new approach in cancer therapy.[12−15]

Most currently available inhibitors of the proteasome exert their inhibitory action by adduct formation with the enzyme.[16−18] The 20S proteasome, which is the catalytic core of the proteasome, is an N-terminal threonine hydrolase.[19] The hydroxyl group on the N-terminal threonine of each β-subunit is prone to react with compounds possessing functional groups receptive to nucleophilic attack. 20S proteasome inhibitors of this type include natural products and synthetic peptides belonging to the following classes of reactive compounds: epoxyketones, aldehydes, boronic acids, α-ketoamides, α-ketoaldehydes, and vinyl sulfones.[20]

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Table 1. IC_{50} Values (μM) in Enzymatic and Cellular Assays

compd	chym[a]	PGPH[a]	tryp[a]	prol[b]	cell[c]
1	0.9	>20	>20		
2	0.007	>20	>20	1.5	
3	0.015	>20	>20	0.06	0.02
4	1.0				
5	0.06	>20	>20	0.9	

[a] Inhibition of chymotrypsin-like (chym), post-glutamyl-peptide (PGPH), and trypsin-like (tryp) proteolytic activity of purified human proteasome. [b] Inhibition of proliferation of MDA-MB-435 cells. [c] Inhibition of chymotrypsin-like activity of the proteasome in cultured MDA-MB-435 cells.

Noncovalent inhibitors of the proteasome seem to have been investigated less extensively judging from the paucity of reports on this type of inhibitor.[21] In principle, such inhibitors should be devoid of the inherent drawbacks associated with the classical reactive warhead groups (i.e., lack of specificity, excessive reactivity, and instability), and we decided to explore this noncovalent strategy in our current drug-discovery activities aimed at blocking the proteasome−ubiquitin pathway in tumor cells.

We have reported in several publications the discovery and optimization of 2-aminobenzylstatine derivatives that inhibit noncovalently and with high selectivity the chymotrypsin-like peptidase activity of the human 20S proteasome (**1** and **2**).[22−24] In this class, we could improve by 2 orders of magnitude the inhibitory activity, in a biochemical assay, of the initial micromolar hit **1**. However, only modest cellular activity could be achieved with this compound class (e.g., derivative **2** in Table 1). Assuming that poor cell penetration was the reason our 2-aminobenzylstatine compounds did not express their high enzymatic inhibitory activity at the cellular level, we engaged in efforts to identify alternative inhibitor scaffolds of reduced size and attenuated peptidic character. We report herein the discovery by structure-based design of the first representatives of a new class of potent, noncovalent 20S proteasome inhibitors that, in contrast to the 2-aminobenzylstatine inhibitors, show high activity in cellular assays.

The active site responsible for the chymotrypsin-like proteolytic activity of the human 20S proteasome is formed by the association of β-subunits X and HC5.[25] Using the X-ray crystal structure of the yeast proteasome,[26] we constructed a homology model of these β-subunits.[23] The model served to establish a binding mode hypothesis for initial compound **1** and was instrumental to the optimization of this class of noncovalent 20S proteasome inhibitors.[24] The binding mode is illustrated in Figure 1 with **2**, one of the most potent noncovalent 20S proteasome inhibitors reported to date.

According to this binding model, which is supported by extensive structure−activity relationships, crucial to the affinity of the 2-aminobenzylstatine inhibitors for the X/HC5 active site is a set of hydrogen bond interactions. Four β-sheetlike hydrogen bonds are formed between the amide bonds flanking the valine residue of the inhibitor and the main chain of subunit X at residues Thr 21, Gly 47, and Ala 49. These hydrogen bonds position the inhibitors in the active site in such

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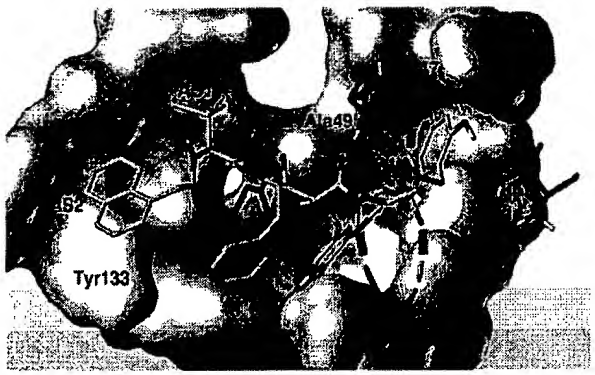


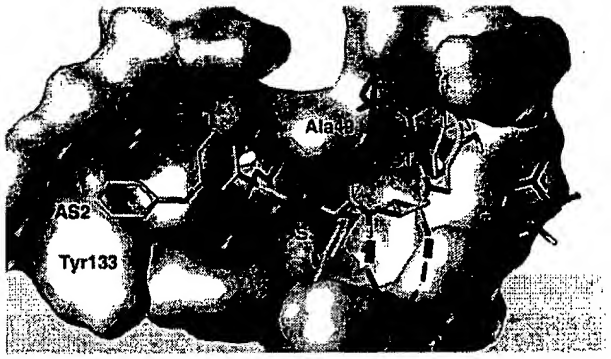
Figure 1. Model of **2** bound to the proteasome X/HC5 subunits. Hydrogen bonds are shown in magenta.

a way that their C-terminal phenol and statine 2-aminobenzyl moieties fill the enzyme's S1 and S3 pockets, respectively, where they form additional hydrophobic and hydrogen bond interactions. In particular, the statine 2-amino group establishes a hydrogen bond with residue Asp 153 of subunit HC5 located on the rim of the S3 pocket. The *tert*-leucine side chain and the lipophilic N-terminal group in the inhibitors also form favorable interactions with the active site by occupying small accessory hydrophobic pockets labeled AS1 and AS2 in Figure 1. In contrast, the two amide bonds flanking the *tert*-leucine residue and the statine 4-benzyl group have no significant interactions with the X/HC5 active site in our binding model. This hypothesis prompted us to envisage the design of new simplified proteasome inhibitor scaffolds in which these nonproductive structural features are removed while maintaining the above-described favorable interactions. Design by interactive molecular modeling led to several ideas for realizing this objective. The most appealing one was a dipeptide scaffold represented by prototype **3**.

Figure 2 shows how the important structural features of the 2-aminobenzylstatine inhibitors are incorporated or mimicked in this molecule. As can be seen, the two crucial amide bonds flanking the valine residue and the C-terminal phenol group filling the S1 pocket are preserved whereas the statine moiety is replaced by a 3,4,5-trimethoxy-L-phenylalanine residue. This nonproteinogenic amino acid is able to form exactly the same interactions with the S3 pocket as the entire 2-aminobenzylstatine moiety. The two other critical pharmacophore features, i.e., the *tert*-leucine side chain and the hydrophobic N-terminal group, are mimicked in the prototype compound by a single phenoxy substituted benzylic N-terminal group. Modeling suggested that the two phenyl rings of this bulky N-terminal group present a spatial arrangement adequate to simultaneously fill the AS1 and AS2 accessory hydrophobic pockets.

Prototypes **4** and **5** (Chart 1), which contrary to **3** do not incorporate all the favorable binding elements present in **2**, were also envisaged for synthesis to further probe the validity of our design concept.

The synthesis of **1** and **2** has been reported previously,[22-24] and **3**–**5** were prepared in a stepwise procedure by standard solution peptide chemistry. The general route for the synthesis of these new 20S proteasome inhibitors is illustrated for **3** in Scheme 1.



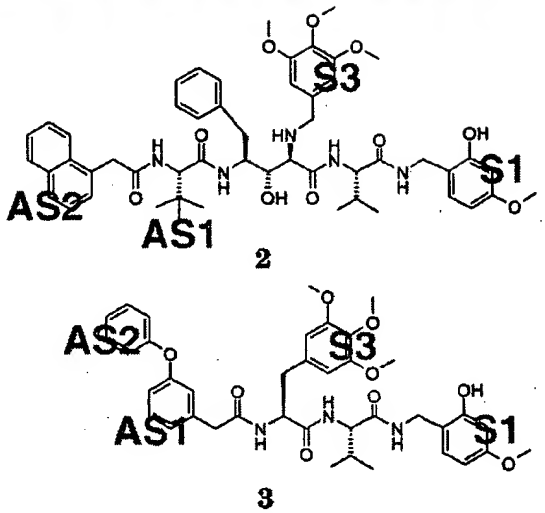
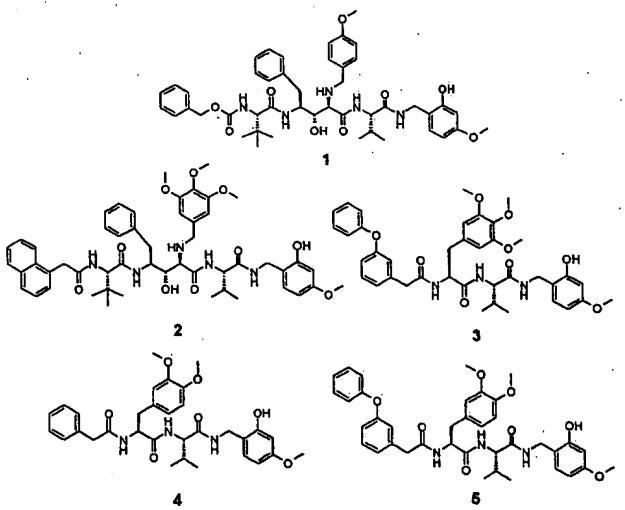


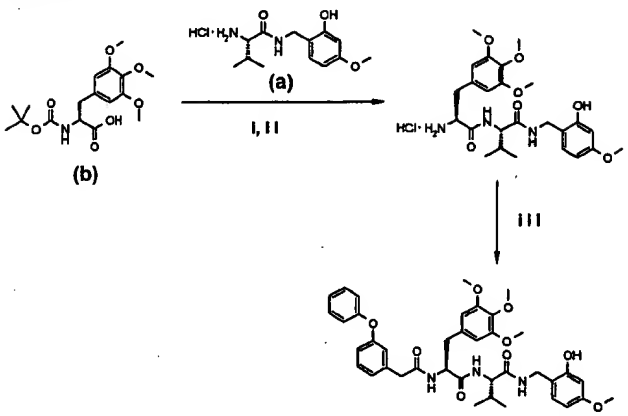
Figure 2. Top: model of designed **3** bound to the proteasome X/HC5 subunits. Hydrogen bonds are shown in magenta. Bottom: chemical structures of **2** and **3** showing the correspondence between their respective chemical moieties in terms of interaction with the proteasome binding site. Matching main chain features are represented in the same color, while matching side chain features are labeled by the name of the pocket to which they bind.

Chart 1



HCl·(*S*)-Val-(2-hydroxy-4-methoxy)benzylamine (**a**) was obtained by condensation of N^{α}-Boc-L-Val-OH with 2-aminomethyl-5-methoxyphenol followed by N^{α}-Boc deprotection with 4 N HCl in dioxane.[22-24] N^{α}-Boc-3,4,5-L-trimethoxyphenylalanine (**b**) was obtained by known

Scheme 1[a]



[a] (i) a, DIEA, TPTU in DMF, 3.5 h, 0 °C, room temp, 65%; (ii) 4 N HCl in dioxane, 90 min, room temp, 100%; (iii) 3-phenoxyphenylacetic acid, DIEA, TPTU in DMF, 16 h, room temp, 77%.

procedures from 3,4,5-trimethoxybenzaldehyde,[27,28] while the 3,4-dimethoxy analogue, N^{α}-Boc-3,4-L-dimethoxyphenylalanine, was from a commercial source. The phenylalanine derivatives were coupled to **a** to provide the Boc-protected dipeptides. After Boc deprotection under acidic conditions, the N-terminal capping groups designed by molecular modeling were incorporated into the corresponding derivatives to afford the target compounds.

The designed prototypes **3**–**5** were tested in biochemical assays that measure their ability to inhibit the three types of hydrolytic activities of the human 20S proteasome. Remarkably, potent and selective inhibition of the proteasome chymotrypsin-like activity was observed. As reported in Table 1, **3** turned out to inhibit the chymotrypsin-like activity with an IC_{50} of 15 nM while not affecting the trypsin-like and post-glutamyl-peptide hydrolytic activities at a concentration as high as 20 μM. Furthermore, fully validating the design concept, the structure–activity relationships observed in the 2-aminobenzylstatine inhibitor class were mirrored in the novel class, as judged from a comparison of the relative inhibitory activities of **3**–**5**. We had observed in the 2-aminobenzylstatine class the marked beneficial effect of filling the AS2 pocket where the main interaction established by the inhibitors, according to the model, is aromatic stacking with residue Tyr 133 of subunit HC5.[23,24] Consistently, the phenoxy substituent targeting the AS2 pocket present on the N-terminal benzylic group of **5** causes a 17-fold increase in potency compared to **4** whose unsubstituted N-terminal group can only interact with the AS1 pocket. Similarly, as previously verified in the 2-aminobenzylstatine series, targeting a residue of the bottom of the S3 pocket[29] for hydrogen bonding by adding a third methoxy substituent on the central benzylic moiety is beneficial. Compound **3** is significantly more potent (4-fold) than **5** in inhibiting the chymotrypsin-like activity of the proteasome.

Thus, overall, the high potency and selectivity of **2** were matched by **3** in biochemical assays. However, most importantly, in contrast to the 2-aminobenzylstatine derivative, **3** also showed good activity in a cellular setting. This compound blocks proteasome activity in cultured cells (IC_{50} = 20 nM) and inhibits in a dose-dependent manner the proliferation of different tumor cell lines (e.g., IC_{50} = 60 nM, MDA-MB-435 cells). Reduction of the molecular weight and decrease of the number of amide bonds thus appear to have been an appropriate strategy to achieve high cellular activity.

In conclusion, we have discovered a new class of potent, cellularly active inhibitors of the proteasome. Unlike most previously reported proteasome inhibitors, the new inhibitors act noncovalently and show high specificity for the chymotrypsin-like activity of the enzyme. These unique properties make **3** an interesting tool for investigations of proteasome function in many aspects of cellular regulation. In addition, the high antiproliferative activity obtained is encouraging in our efforts toward developing an anticancer drug based on the concept of proteasome inhibition.

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Supporting Information Available: Experimental procedures and analytical data for all intermediate and final compounds and description of biochemical and cellular assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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(27) *N*-Acetyl-3,4,5-trimethoxy-L,D-phenylalanine was prepared from commercially available 3,4,5-trimethoxybenzaldehyde and *N*-acetylglycine according to a literature procedure (Oltz, E. M.; Bruening, R. C.; Smith, M. J.; Kustin, K.; Nakanishi, K. The Tunichromes. A Class of Reducing Blood Pigments from Sea Squirts: Isolation, Structures, and Vanadium Chemistry. *J. Am. Chem. Soc.* **1988**, *110*, 6162−6172). Alternatively, the *N*-acetyl-3,4,5-trimethoxy-L,D-phenylalanine methyl ester was obtained from 3,4,5-trimethoxybenzaldehyde and malonic acid dimethyl ester following known protocols (see experimental section).
(28) The resolution of the racemic *N*-acetyl-3,4,5-trimethoxy-L,D-phenylalanine methyl ester was performed by enzyme-catalyzed hydrolysis of the L-ester using Alcalase (Novo Nordisk) as described in the literature. Nestor, J. J., Jr.; Ho, T. L.; Simpson, R. A.; Horner, B. L.; Jones, G. H.; McRae, G. I.; Vickery, B. H. Synthesis and Biological Activity of Some Very Hydrophobic Superagonist Analogues of Luteinizing Hormone-Releasing Hormone. *J. Med. Chem.* **1982**, *25*, 795−801. Tyagi, O. D.; Boll, P. M. Synthesis of (*S*)-3,4-dihydroxyphenylalanine (L-DOPA) and unnatural α-amino acids via enzymatic resolution using alcalase. *Indian J. Chem.* **1992**, 851−854.
(29) This residue can be Tyr 135 or Ser 157 of subunit HC5. See ref 24.

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